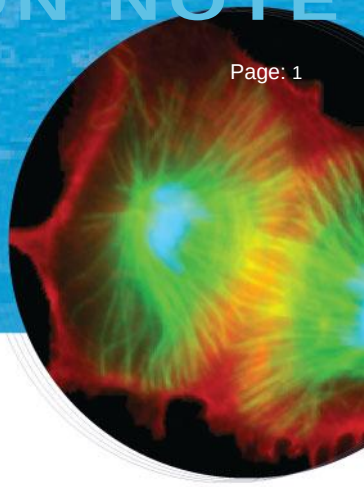
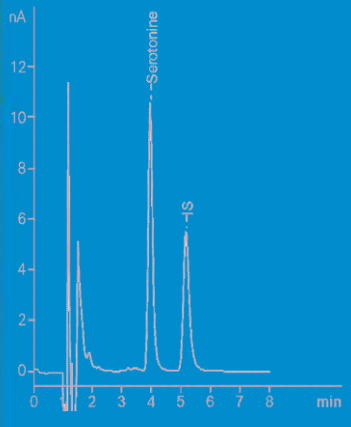
COMPILATION OF CLINICAL APPLICATIONS



THE SOUNDEST LC-EC APPLICATIONS FOR CLINICAL & DIAGNOSTICS ANALYSIS EVER BUILD

Catecholamines
Serotonin
Metanephrines
VMA
HVA
5-HIAA
Homocysteine
Glutathione
(di-)sulfides
Iodide
Vitamins A, C, D, E, and K
Q10
Ubiquinols



INTRODUCTION

Analysis of bodily fluids in clinical laboratories is of great diagnostic importance to discover diseases in an early stage. A number of tumor and cardiovascular markers have been analyzed using dedicated ALEXYS Analyzers. Special attention has been paid to sample throughput, reliability and robustness of analysis. The measurement of catecholamines and serotonin in biological fluids is routinely performed for the diagnosis of tumours such as neuroblastomas and ganglioneuroblastomas. Clinical and diagnostic labs are required to test more urine and plasma samples, more accurately, more efficiently. With its dedicated Analyzers Antec helps you to meet those needs routinely and with confidence.

- ALEXYS Analyzers in Clinical Analysis
- Optimized for performance
- Dedicated system solutions

Summary

A selection is presented of different application notes from the work of a few of our many users. These notes demonstrate the versatility of our analyzers in different experimental conditions.

Contents

Fig. 1. ALEXYS Clinical Analyzer.

Serotonin in human plasma

Introduction

Within the circulation more than 99% of serotonin (5HT) is stored in blood platelets. Increased concentrations in circulating 5HT have been implicated in migraine, schizophrenia, carcinoid syndrome, essential hypertension, Huntington's chorea and Duchenne's dystrophy (see 1). In addition, changes in 5HT function in platelets seem to reflect central nervous system functions of 5HT, so platelet 5HT function is also proposed as a peripheral model for 5HT in the CNS (2).

LCEC provides a highly sensitive and specific means to analyse the fate of 5HT in biological matrices. However, collection and the preparation of blood samples without disturbing the massive but very delicate platelet pool requires great care to prevent leakage from this pool (2, 3). This can be accomplished by centrifuging at a low and high speed subsequently to separate the platelets from the remainder of the blood without disturbing the platelet integrity.

Method

Venous blood samples (10 ml) are collected from the cubital vein in EDTA tubes (10 ml, 0.12 ml EDTA, 15%). Samples are centrifuged (90 g) at 4°C during 20 min and the supernatant, containing platelet-rich plasma is transferred to 8 ml tubes and centrifuged again (2500 g) at 4°C during 20 min. The supernatant is transferred and frozen (-20°C). Prior to analysis the samples are thawed, diluted 1.4 times with HPLC buffer without ion-pairing agent, the internal standard N-methyl-5HT(nMet), 10 µl, 0.22 µg/ml and TCA (65 µl, 3.1 M) are added and this mixture is centrifuged (1500 g, 20 min, 4°C) . A volume of 20 µl of the supernatant is injected into the LCEC system.

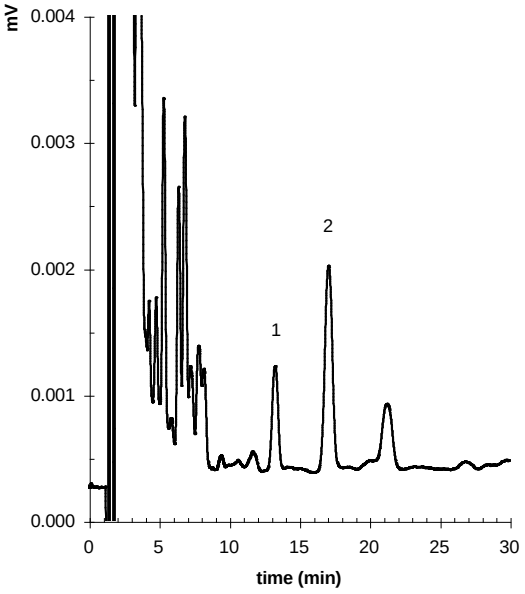


Fig. 1. Chromatogram of 5-HT (1) and nMet (2, IS) in human plasma. Concentration is 1 ng/ml. Courtesy: J.C.M. Schouten - Verhagen et al., Rijngeest Group, Oegstgeest.

References

1. P.C. Tagari, D.J. Boullin and C.W. Davies, Clin. Chemistry 30 (1984) 131 – 135.
2. C.M. Middelkoop, G.A. Dekker, A.A. Kraayenbrink, and C. Popp-Snijders, Clin. Chem., 39, 1993, 1675-1678.
3. M. Picard, D. Olichon and J. Gombert, J. Chromatogr. 341, 1985, 445-451.

Table 1

Set-up	
Column	Merck Superspher Select B (125 x 4 mm ID), pre-column Select B (10 mm)
Flow rate	0.7 ml/min
Mobile phase	50 mM citrate, 50 mM acetate (pH 4.0), 0.96 mM SOS, 0.54 mM EDTA and 4.5% ACN (v/v)
Temperature	30°C
Icell	0.1 – 0.5 nA
Flow cell	VT-03, 3 mm glassy carbon WE
E-cell	600 mV vs. Ag/AgCl sat'd

Recommendation

The advised configuration for this application is the ALEXYS Analyzer using an autosampler with sample cooling option.

PART NUMBERS AND CONFIGURATION	
180.0035C	ALEXYS Analyzer – cooled
110.4105	VT03 flow cell, 3 mm GC, sb

Free catecholamines in urine

Introduction

The catecholamines noradrenaline (NA) and adrenaline (A) play an important role in sympathetic activity, and sympathetic dysfunction may be reflected in variable changes in catecholamine production and excretion (1). In clinical laboratories they are measured mainly for the diagnosis and follow-up of catecholamine producing tumours like phaeochromocytoma and neuroblastoma and, to a lesser extent, also to diagnose orthostatic hypotension. Although phaeochromocytomas are usually benign, confirmation of diagnosis is important because of the harmful effects of increased circulating catecholamines, such as severe hypertension. The definitive diagnosis of phaeochromocytoma rests primarily on the measurement of catecholamines in blood or urine, or of their urinary metabolites (see Application Notes 213-008 and 213-004). LCEC is known to be a fast and reliable method for the analysis of urinary catecholamines and metabolites.

Method

An equilibrated ion exchange column is used for each sample. The pH of the urine is adjusted to pH 6.5 by NaOH. EDTA (0.1%) and the internal standard (dihydroxybenzylamine, DHBA, 0.01 mg/ml) solution are added to 3.0 ml urine and this sample is applied to the cation exchange column (Biorad, part no. 1956017). Possible interferences are removed by rinsing the columns with water and the catecholamines are eluted with boric acid (4% W/V). A 20 µl aliquot of the sample is injected and separation and quantitation are achieved by a reversed phase column and an electrochemical detector respectively. The recovery percentage is calculated by comparing the peak areas of the internal standard DHBA added to the urine sample with a standard sample of DHBA also comprising NA, A and DA ('external standard'). It is assumed that the recovery percentages for NA, A and DA are the same as for DHBA and hence this loss is corrected for all 3 catecholamines.

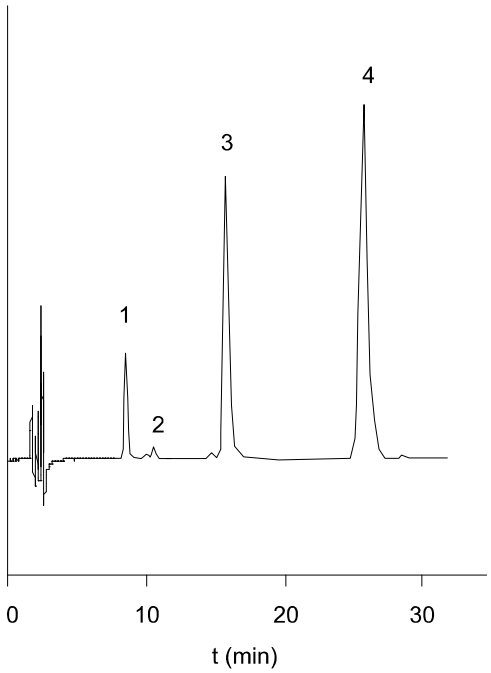


Fig. 1 Analysis of 190 nmol/l NA (1), 23 nmol/l A (2), 83 nmol/l DHBA (3) and 1.1 µmol/l DA (4).
Courtesy: Dr. C. Popp-Snijders, Endocrine laboratory, Free University Hospital, Amsterdam, The Netherlands.

References

1. B. Kagedal and D.S. Goldstein, J. Chromatography 429 (1988) 177-233.
2. P.E. Graham, G.A. Smithe, G.A. Edwards and L. Lazarus, Ann. Clin. Biochem. 30 (1993) 129-134.

Table 1

Set-up	
Column	Supelco LC-18, 150 x 4.6 mm, 3 µm
Flow rate	1.0 ml/min
Sample	20 µl
Mobile phase	Chromsystems catecholamine buffer
Temperature	30°C
E-cell	600 mV (vs. Ag/AgCl sat'd KCl)

Recommendation

The advised configuration for this application is the ALEXYS Analyzer using an autosampler with sample cooling option.

PART NUMBERS AND CONFIGURATION	
180.0035C	ALEXYS Analyzer – cooled
110.4105	VT03 flow cell, 3 mm GC, sb

Total catecholamines in urine

Introduction

The catecholamines noradrenaline (NA) and adrenaline (A) play an important role in sympathetic activity, and sympathetic dysfunction may be reflected in variable changes in catecholamine production and excretion (1). In clinical laboratories they are measured mainly for the diagnosis and follow-up of catecholamine producing tumours like phaeochromocytoma and neuroblastoma and, to a lesser extent, also to diagnose orthostatic hypotension. Increased levels of catecholamines are associated with hypertension, however, phaeochromocytomas are responsible for only 0.1 % of cases of hypertension. Although usually benign, confirmation of diagnosis is important because of the harmful effects of increased circulating catecholamines. The definitive diagnosis of phaeochromocytoma rests primarily on the measurement of catecholamines in blood or urine, or of their urinary metabolites (see Application Notes 213-008 and 213-004).
LCEC is known to be a fast and reliable method for the analysis of urinary catecholamines and metabolites.

Method

Prior to extraction on an equilibrated ion exchange column urine samples (about 12 ml, acidified with HCl conc to pH 1) are hydrolysed by boiling (100°C). After hydrolysis the pH of the urine is adjusted to pH 6.5 by NaOH. EDTA (0.1%)and the internal standard (dihydroxybenzylamine, DHBA, 0.01 mg/ml)) solution are added to 3.0 ml urine and this sample is applied to a cation exchange column (Biorad 1956017). Possible interferences are removed by rinsing the columns with water and the catecholamines are eluted with boric acid (4% W/V A 20 µl aliquot of the sample is injected in duplicate and separation and quantitation are achieved by a reversed phase column and an electrochemical detector resp. The recovery percentage is calculated by comparing the peak areas of the internal standard DHBA added to the urine sample with a standard sample of DHBA also comprising NA, A and DA ('external standard'). It is assumed that the recovery percentages for NA, A and DA are the same as for DHBA and hence this loss is corrected for all 3 catecholamines.

Table 1

Set-up	
Column	Supelco LC-18, 150 x 4.6 mm, 3 µm
Flow rate	1.0 ml/min
Sample	20 µl, extracted with Chromsystems ion exchange columns
Mobile phase	Chromsystems catecholamine buffer
Temperature	30°C
E-cell	600 mV (vs. Ag/AgCl sat'd)
I-cell	ca. 0.8 nA

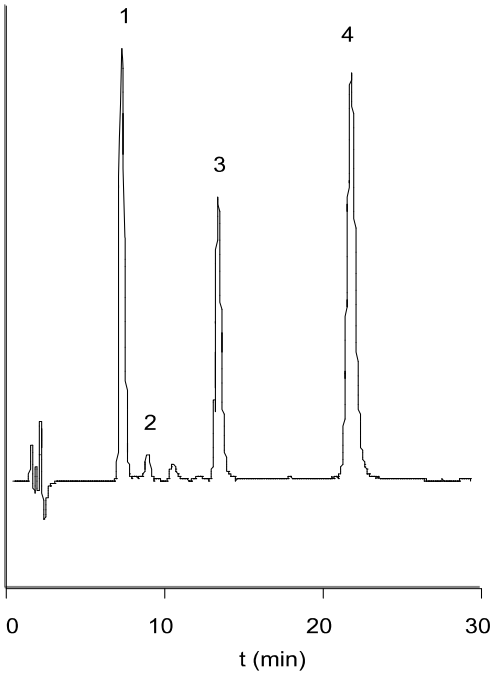


Fig. 1 Analysis of 190 nmol/l NA (1), 23 nmol/l A (2), 83 nmol/l DHBA (3) and 1.1 µmol/l DA (4).
Courtesy: Dr. C. Popp-Snijders, Endocrine laboratory, Free University Hospital, Amsterdam, The Netherlands.

References

1. B. Kagedal and D.S. Goldstein, J. Chromatography 429 (1988) 177-233.
2. P.E. Graham, G.A. Smithe, G.A. Edwards and L. Lazarus, Ann. Clin. Biochem. 30 (1993) 129-134.
3. I.J. Kopin, Pharmacol. Reviews 37 (1985), 333-364.

Recommendation

The advised configuration for this application is the ALEXYS Analyzer using an autosampler with sample cooling option.

PART NUMBERS AND CONFIGURATION	
180.0035C	ALEXYS Analyzer – cooled
110.4105	VT03 flow cell, 3 mm GC, sb

VMA, HVA and 5-HIAA in urine

Introduction

The catecholamines dopamine, noradrenaline and adrenaline have a variety of important physiological functions in the central and peripheral nervous system. They are primarily inactivated by (re-)uptake into the nerve endings. Up to 90% of the originally released neurotransmitter is recaptured in this fashion. Catecholamines entering the plasma are degraded rapidly by catechol-O-methyl transferase to metanephrines. The further metabolic pathway via monoamineoxidase and aldehyde dehydrogenase leads to the end products vanillylmandelic acid (VMA) and homovanillic acid (HVA).

Tumour diseases of catecholamine synthesising tissue or catecholamine dependent tissue of the nervous system are associated with increased production of the neurotransmitters resulting in characteristic changes in the urinary excretion profile of the metabolites.

Determination of the serotonin metabolite 5-hydroxy-indole-acetic acid (5-HIAA) is particularly relevant for the diagnosis of carcinoid syndrome, a tumour of the enterochromaffin cells in the small intestine which leads to an increased serotonin production.

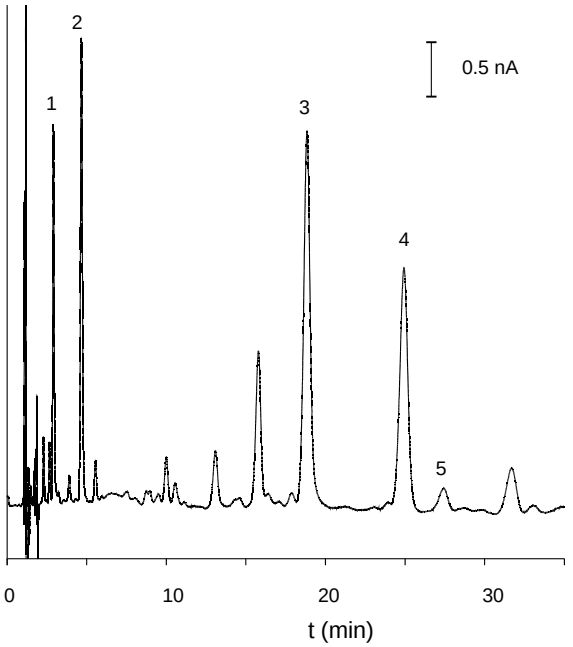


Fig. 1. Analysis of VMA, HVA and 5-HIAA in human urine. Peaks are: 1: VMA (26.7.µmol/l), 2: iso-VMA (IS), 3: 5-HIAA (66.4 µmol/l), 4: 5-HICA (5-hydroxyindolecarboxylicacid, IS), and 5: HVA (66.4 µmol/l).
Courtesy: Dhr. J. van der Kolk, Clin. Chem. Lab., Bethesda Hosp., Hoogeveen, The Netherlands.

Method

The sample pre-treatment consists of a liquid-liquid extraction with back extraction into phosphate buffer. Urine samples (5 ml) are acidified by adding 50 µl concentrated acetic acid. A volume of 0.5 ml acidified urine is mixed with 0.5 ml water, 0.5 ml internal standard (iso-VMA and 5-HICA, 70 nmol/l) and 1 g of NaCl. After addition of 2.5 ml ethylacetate, the mixture is vigorously shaken and centrifuged. A volume of 2 ml of the organic layer is transferred to a clean tube and 2 ml of phosphate buffer (200 mmol/l, pH=7) is added. After vigorously shaking and centrifugation the organic layer is removed and 50 µl of the aqueous layer is added to 3 ml of mobile phase. An aliquot of 20 µl is used for injection into the HPLC.

Table 1

Set-up	
Column	PhaseSep S3 ODS2, 100 x 4.6 mm, 3 µm, 20 µl injection
Flow rate	1.0 ml/min
Mobile phase	Phosphoric acid 100 mM, citric acid 100 mM, pH 3.0 with KOH, EDTA 20 mg/l, octane sulphonic acid 400 mg/l, MeOH 6%
Sample	liquid-liquid extract of 24 h urine
Temperature	ambient
E-cell	900 mV (vs. Ag/AgCl sat'd)
I-cell	ca. 10 nA

Recommendation

The advised configuration for this application is the ALEXYS Analyzer using an autosampler with sample cooling option.

PART NUMBERS AND CONFIGURATION	
180.0035C	ALEXYS Analyzer – cooled
110.4105	VT03 flow cell, 3 mm GC, sb

Plasma catecholamines

Introduction

The catecholamines dopamine, noradrenaline and adrenaline play an important role in information transmission and regulation of metabolism in the body. Quantitative analysis of catecholamines is diagnostically important in a number of disease states such as hypertension, Parkinson's disease, schizophrenia, epilepsy etc. Of all detection methods available for the determination of catecholamines, HPLC with electrochemical detection is one of the most widely used. It is highly sensitive, selective and easy to apply.

Method

Samples of biological origin require a purification step before HPLC analysis. Often used pre-treatment procedures are ion-exchange, extractions using alumina (Al_2O_3) or diphenyl borate ethanolamine (DPBEA). A number of methods have been developed and have been reviewed extensively [1, 2].

In this application a DPBEA extraction is used. The principle of this extraction is based on a complex formation between borate and the catecholamine diol at alkaline pH. DPBEA is dissolved in phosphate buffer at pH 8. The resulting ion pair is extracted in an organic phase (heptane). This extraction step is followed by a second extraction into an aqueous phase (80 mM acetic acid), and injection into an HPLC system.

The HPLC system consists of a reversed phase column with an ion-pair added to the mobile phase (Table I). The retention of polar amines such as noradrenaline is selectively affected by the ion-pair concentration, acidic sample constituents are selectively affected by the pH, while the modifier percentage affects both. Optimisation of resolution is therefore done by variation of these three parameters.

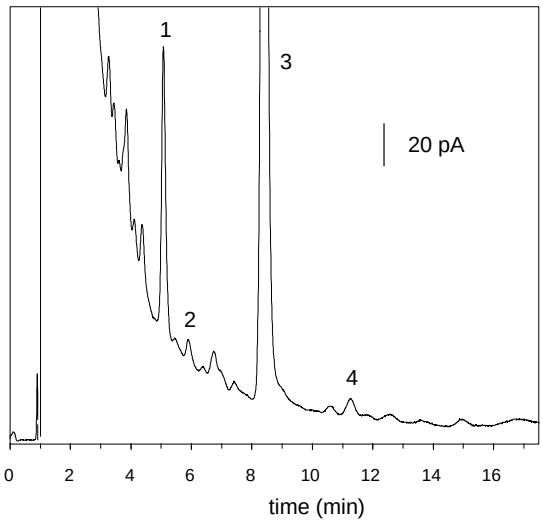


Fig. 1. Analysis of human plasma after liquid-liquid extraction with DPBEA/Heptane. On-column amounts are: 1. norepinephrine: 31 pg, 2. epinephrine: 2.9 pg, 3. DHBA (IS): 200 pg, 4. dopamine: 1.5 pg. Plasma concentrations are: norepinephrine 386 pg/ml, epinephrine: 36 pg/ml, dopamine: 15 pg/ml. Courtesy: Ing. G. Alberts, Dept. of Int. Med. I, Univ. Hosp. 'Dijkzigt', Rotterdam, The Netherlands.

References

1. F. Ponzio, G. Achilli and C. Perego, in Electrochemical detection in medicine and chemistry (Ed. Parvez et al.),p. 307 - 329, 1987 VNU Science Press, Utrecht, The Netherlands.
2. E. Gelpí, in Advances in Chromatography (Eds. J.C. Giddings, E. Grushka and P.R. Brown), vol. 26 (1987) 321 – 391

Table 1

Set-up	
Column	C18, 3 µm, 100 x 2.0 mm
Flow rate	0.2 ml/min
Mobile phase	Ammonium acetate 50 mM, glacial acetic acid 1.25%, sodium dodecyl sulphate 0.347 mM, EDTA 0.27 mM, 25% methanol
Injection	20 µl
Temperature	32.5 °C
E-cell	600 mV (vs. Ag/AgC, satd')

Recommendation

The advised configuration for this application is the ALEXYS Analyzer using an autosampler with sample cooling option.

PART NUMBERS AND CONFIGURATION	
180.0035C	ALEXYS Analyzer – cooled
110.4105	VT03 flow cell, 3 mm GC, sb

Metanephrines in urine

Introduction

Normetanephrine, metanephrine and 3-methoxytyramine are degradation products of noradrenaline, adrenaline and dopamine respectively. These metanephrines are formed by methylation of catecholamines by the enzyme catechol-o-methyltransferase. The metabolites are released into the blood stream and excreted mainly via the kidneys. The urinary excretion of catecholamines and their metabolites is an index of the activity of the sympathetic nervous system and allows diagnostic inferences in certain diseases.

The principal diagnostic test for diseases such as pheochromocytoma, neuroblastoma and ganglioneuroma, is measurement of urinary catecholamines and the acidic metabolites vanillylmandelic acid and homovanillic acid. However for reliable diagnosis further differential investigations are essential. Including the excretory profile of the metanephrines in the diagnostic consideration of nervous system tumours decreases the incidence of false negative results.

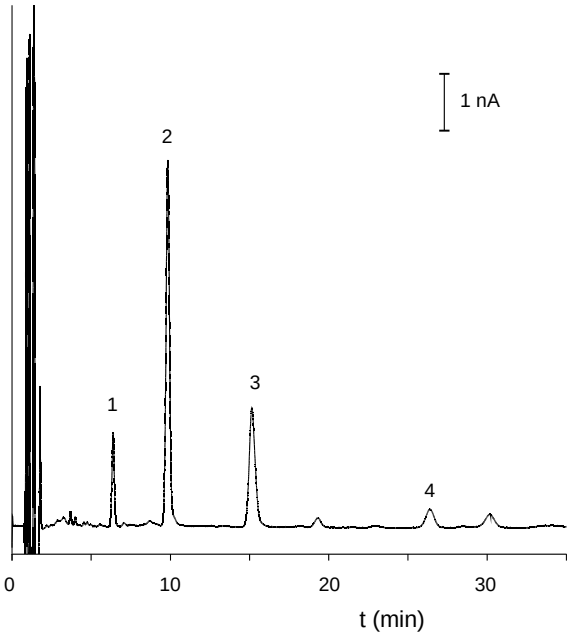


Fig. 1. Analysis of human urine. Peaks are: 1: normetanephrine (0.9 µmol/l), 2: metanephrine (8.2 µmol/l), 3: hydroxymethylbenzylamine (IS), and 4: 3-methoxytyramine (0.5 µmol/l). Courtesy: Dhr. J. van der Kolk, Clin. Chem. Lab., Bethesda Hosp., Dr. G.H. Amshoffweg 1, 7909 AA Hoogeveen, The Netherlands.

Method

The major fraction of the metanephrines is excreted in conjugated form, either as glucuronides or sulphates. Since sample preparation on an ion exchange column isolates only the unconjugated forms, prior acid hydrolysis is essential.

After addition of internal standard, urine samples are acidified and hydrolysed by boiling. Metanephrines are isolated by ion exchange chromatography and analysed by ion pair chromatography with electrochemical (EC) detection [1].

References

1. R.N. Gupta, Clin. Chem. 36 (1990) 538-540.

Table 1

Set-up	
Column	PhaseSep S3 ODS2, 100 x 4.6 mm, 3 µm, 20 µl injection
Flow rate	0.80 ml/min
Mobile phase	Phosphoric acid 100 mM, citric acid 100 mM, pH 3.0 with KOH, EDTA 20 mg/l, octane sulphonic acid 400 mg/l, MeOH 6%
Sample	acid hydrolysate of 24 h urine (cation exchange + anion exchange extract)
Temperature	ambient
E-cell	875 mV (vs. Ag/AgCl, sat'd)
I-cell	ca. 5 nA

Recommendation

The advised configuration for this application is the ALEXYS Analyzer using an autosampler with sample cooling option.

PART NUMBERS AND CONFIGURATION	
180.0035C	ALEXYS Analyzer – cooled
110.4105	VT03 flow cell, 3 mm GC, sb

Iodide in urine

Introduction

A method for the determination of iodide is developed using electrochemical detection (ECD) with a silver working electrode (WE). The method consists of ion exchange chromatography using a phosphate/citrate buffer at pH 6.5, followed by amperometric detection at 0.15 V.

Detection limit of iodide is 0.2-1 µmol/L (25 - 127 ng/mL) depending on the column performance. Reproducibility of the determination of iodide is concentration depended. At 50 µM RSD in peak area's and heights was better than 2%. Below 1 µM the RSD values increased to 10 – 22 %.

Method

The HPLC system consists of a anion exchange column with a phosphate/citrate mobile phase (Table I). The use of halide ions such as chloride and bromide must be avoided as they are reactive towards the silver electrode, causing a high background current and decreased sensitivity.

Di-sodium phosphate and citric acid (both 10 mM) are dissolved in 800 mL water, pH is set to 6.5 with NaOH and water is added to 900 mL. Finally 10% methanol (100 mL) is added.

Determination of analytes in complex matrices such as urine or plasma is often complicated by sample pre-treatment procedures to improve the selectivity of a method. Without suitable pre-treatment co-eluting peaks make reliable and reproducible analysis impossible.

An exception is the determination of iodide in urine. Due to the selectivity of the silver working electrode, urine could be injected directly. After dilution and filtration over a 0.2 µm membrane filter, urine samples have been injected onto the analytical column and analysed.

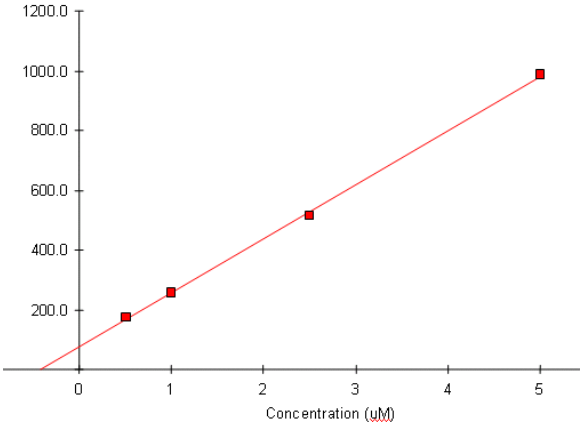


Fig. 1. Regression line of standard addition analysis of iodide in diluted and filtrated urine (see results in Fig. 2).
Line: Y = 76.6 + 180.9B, r=0.9996.

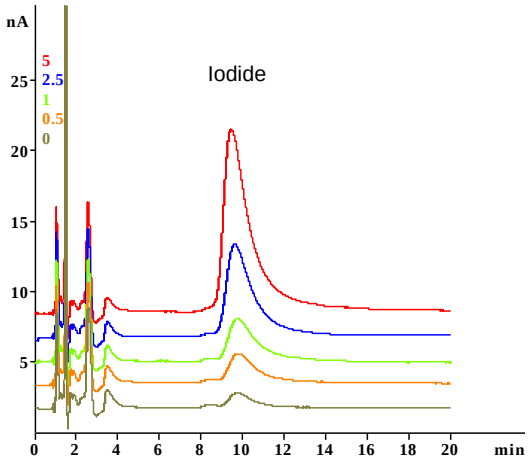


Fig. 2. Standard addition analysis of iodide in diluted (factor 10) and filtrated urine using ECD with a silver working electrode (20 µL). From top to bottom: urine with addition of 5, 2.5, 1, 0.5 and 0 µmol/L potassium iodide. Peak height of iodide (at t = 10 min) corresponds to a concentration of 4 µmol/l in urine.

Table 1

Set-up	
Column	ALD-510 anion exchange column, 100 x 4.6mm, 7um
Flow rate	1.3 mL/min
Mobile phase	Na2HPO4 and citric acid (both 10 mM), pH set to 6.5 with NaOH, 10 % MeOH.
Temperature	35 ºC (separation & detection)
Sample	urine is diluted (10x) and membrane filtrated (0.2 µm filter)
Range	500 nA/V
I-cell	30 - 50 nA
E-cell	150 mV vs HyREF

Recommendation

The advised configuration for this application is the ALEXYS Analyzer using an autosampler with sample cooling option.

PART NUMBERS AND CONFIGURATION	
180.0077C	ALEXYS Iodide Analyzer

Homocysteine in plasma

Introduction

Homocysteine is a strongly oxidising and toxic product of amino acid metabolic pathways and raised blood levels (homocysteinaemia) are an established risk factor for arteriosclerosis and coronary heart disease. Increases of 10-15 % in the level of homocysteine raise the risk of coronary heart disease and cerebral vascular incidents approximately 3 to 4 fold. The current HPLC methods, relying on derivatisation of homocysteine and fluorescence detection, are laborious and the fluorescence label is quite expensive.

Therefore, a highly sensitive, economical, reproducible and rapid method for the analysis of homocysteine in plasma by isocratic reversed phase LC with electrochemical detection (EC) has been developed. The detection limit with standards is 0.3 nmol/L, RSD in peak heights is better than 1% and homocysteine is eluted within 5 minutes. Recovery from plasma samples is nearly 100%.

Method

About 70 % of homocysteine in plasma is bound to albumine, 30% is present as mono- or disulphide. Therefore, prior to determination of total homocysteine, disulphides are reduced and released from albumine by TBP (tri-n-butylphosphine).

The internal standard N-(2-mercapto propionyl)glycine (2MPG, 50 µL of 16 mg/L), and 50 µL of TBP are added to 200 µL of plasma. After 30 min of incubation at 4 ºC 200 µL of trichloroacetic acid (TCA) is added, and vigorously shaken for 2 min. After incubation for 15 min at 4 ºC, the mixture is centrifuged at 10,000 RPM for 10 min. A volume of 100 µL of supernatant is diluted to 5 mL with mobile phase and analysed by LC-EC.

A working potential of +600 mV is applied. Prior to each run a cleaning pulse of +1 V and -1 V both of 1 s is applied, followed by 5 minutes stabilisation. The linearity has been checked using concentrations of 20, 40, 60, 80 and 100 nmol/L. The detector response is linear in this range with a regression coefficient better than 0.998 and with a detection limit of 0.3 nmol/L.

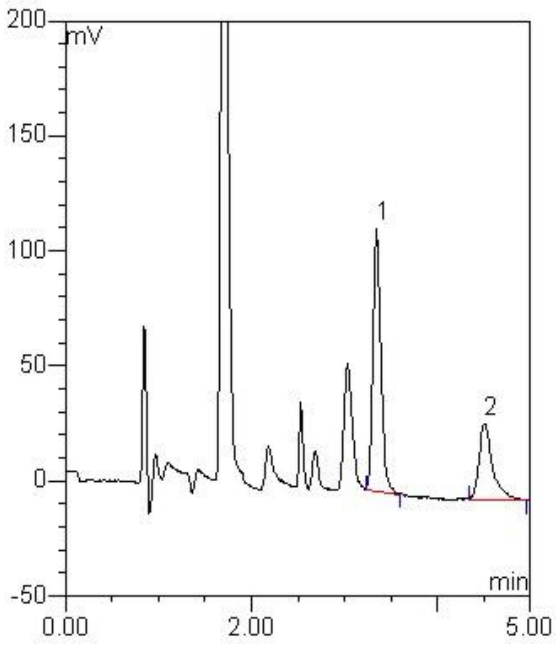


Fig. 1. Chromatogram of 39 µmol/L homocysteine (1) and 98 µmol/L 2MPG (2) in plasma. Range setting is 5 nA/V. Courtesy: J. v.d. Kolk, Bethesda hospital, Hoogeveen [1].

References

1. Determination of homocysteine, Standard operating procedure A.29 HCYS_1, Bethesda Hospital, Hoogeveen, The Netherlands
2. Nicola C. Smith, Mark Dunnet, Paul C. Mills, Journal of Chrom. 673 (1995), 35-41.
3. G.H.J. Boers, Mediator 8(2), March 1997, 9-11

Table 1

Set-up	
Column	Spherisorb S3 ODS2, 4.6 * 100 mm, 3 µm
Flow rate	1 mL/min
Sample	20 µL
Mobile phase	0.15 mol/L phosphoric acid (1%), 2 mmol/L KCl, 10 mg/L OSA pH 1.75 (with NaOH).
Temperature	30°C
E-cell	600 mV (vs. ISAAC 2 mM KCl)
I-cell	1.90 nA

Recommendation

The advised configuration for this application is the ALEXYS Analyzer using an autosampler with sample cooling option.

PART NUMBERS AND CONFIGURATION	
180.0035C	ALEXYS Analyzer – cooled
102.4325	Flexcell Au HyREF

Ascorbic acid in human plasma

Introduction

Ascorbic acid (AA) is normally present in human as well as in animal plasma and tissue. The daily need is considered to be at least 70 mg and normal blood levels are between 28 and 85 µM. For example, the analysis of AA in plasma of geriatric patients reflects the quality of feeding status of such patients. Since it is quite labile, accurate measurement of AA in biological matrices highly depends on a reliable method to stabilise AA (1, 2). So, if samples are stored without specific precautions auto-oxidation of AA may occur, even after 15 minutes, and erroneous measurements will result. It appears that dithiothreitol (DTT) and meta-phosphoric acid (MPA) are quite effective as an aid for long-term stabilisation (up to one year) of AA in biological samples and, in addition, DTT appears to reduce dehydro-ascorbic acid (1, 2). The analysis with LCEC is very straightforward and highly reproducible with the use of hydrochinon as internal standard. The runtime is determined by DTT eluting after about 15 min in the current phase system whereas AA already elutes after less than 3 min. Uric acid is usually present in the chromatograms but does not interfere.

Method

Blood samples of at least 1 ml are centrifuged and 0.2 ml of the supernatant is thoroughly mixed with 0.9 ml MPA (3%) and immediately frozen (-20°C). After thawing, 0.2 ml phosphate buffer (pH 7.4) with a known amount of DTT is added, mixed and left for 30 min. If samples are not stored 0.2 ml sample is mixed with 0.2 ml of the phospate/DTT mixture and left for 30 min. Then these non-stored samples are mixed with 0.9 ml MPA 3%. Subsequently, both types of samples are centrifuged (5 min, 10,000 g), 0.1 ml is transferred to the LC sample vials containing 0.9 ml MPA 3% and mixed. A volume of 10 µl of this mixture is injected into the LCEC system.

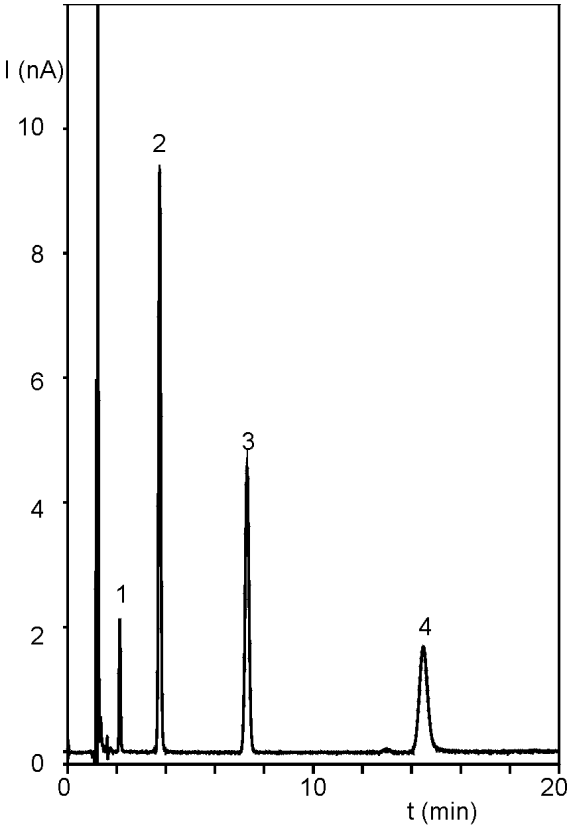


Fig. 1 Chromatogram of ascorbic acid (1) in pretreated plasma. Plasma concentration is 41 µmol/l. Other peaks are: Urate (2), Hydrochinon (IS, 3) and DTT (4). Courtesy: M. van der Horst, M.G.J. Lunenborg and E. Oeben, Scheper Ziekenhuis, Emmen, The Netherlands.

References

1. S.A. Margolis and T.P. Davis, Clin. Chemistry, 34 (1988) 2217 – 2223.
2. S.A. Margolis, R.C. Paule and R.G. Ziegler, Clin. Chemistry, 36 (1990) 1750 – 1755.

Table 1

Set-up	
Column	Supelco C18-BDS, 5 µm, 150 x 4.6 mm
Flow rate	1.3 ml/min
Mobile phase	Phosphate buffer (50 mM), pH 2.8, 200 mg/l EDTA, 2% methanol (v/v)
Sample	10 µl inj.
Temperature	30°C
I cell	ca. 0.4 nA
E-cell	600 mV vs. Ag/AgCl sat'd

Recommendation

The advised configuration for this application is the ALEXYS Analyzer using an autosampler with sample cooling option.

PART NUMBERS AND CONFIGURATION	
180.0035C	ALEXYS Analyzer – cooled
110.4105	VT03 flow cell, 3 mm GC, sb

Vitamin-K in plasma

Introduction

Vitamin K is a fat soluble vitamin involved in several biochemical processes as a cofactor in carboxylation reactions. An example is the clotting of the blood.

Vitamin K is necessary for the proper formation of the blood plasma protein prothrombin, the inactive precursor of thrombin. This enzyme converts the protein fibrinogen of blood plasma into fibrin, the insoluble, fibrous protein that holds blood clots together. The K vitamins are a group of several closely related molecules, consisting of a naphthoquinone skeleton with an isoprene side chain of differing length.

Method

Several assays have been described for the analysis of vitamin K. The K vitamins occur at very low concentrations in plasma (0.1 - 4 ng/ml). Therefore, a sensitive detection method such as EC detection is required. To improve the selectivity of the method, a 2 step sample pre-treatment is used consisting of a liquid-liquid extraction (LLE) followed by a solid phase extraction (SPE). Before EC detection the quinone moiety is converted into a quinol, by a post-column reactor flow cell.

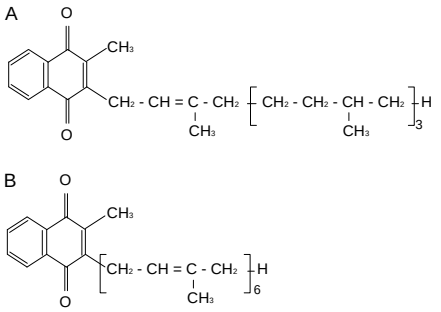


Fig. 1 Two major forms of vitamin K. (A) Vitamin K1 (phylloquinone) is found in plants. (B) Vitamin K2 (menaquinone-6) is found in animals.

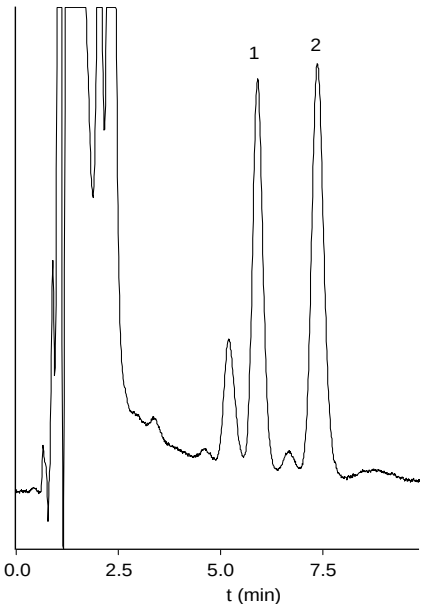


Fig. 2 Analysis of vitamin K1 (1) in plasma extract. Concentrations are 2.5 ng/ml for vitamin K1 and 5 ng/ml for internal standard 2,3 hydrophylloquinon.

References

1. M.J. Shearer, Adv. in Chromatogr. 21 (1983) 243 - 301
2. J.P. Langenberg and U.R. Tjaden, J. Chromatogr. 305 (1984) 61 – 72

Table 1

Set-up	
Column	Higgins Spherisorb ODS2 50 x 2.1 mm, 5 µm
Flow rate	0.2 ml/min
Sample	plasma after LLE, SPE, evaporation and reconstitution in mobile phase water
Mobile phase	0.1 M lithiumperchlorate in methanol with 4%
Temperature	30 °C
E-cell	reactor: -500 mV, detection: 300 mV (vs. HyREF)

Recommendation

The advised configuration for this application is the ALEXYS Analyzer using an autosampler with sample cooling option.

PART NUMBERS AND CONFIGURATION	
180.0035C	ALEXYS Analyzer – cooled
110.4305	VT03 flow cell, 3 mm GC, HyREF

Q10, ubiquinols, vitamin E, and ß-carotene in human LDL

Introduction

Coenzyme Q10 is one of a number of naturally occurring ubiquinones that act as electron acceptors in mitochrondrial oxidative phosphorylation. The reduced forms, the ubiquinols, are strong anti-oxidants. Also, oxidation of LDL (low-density lipoproteins) is believed to play an important role in early atherosclerosis. According to this oxidation hypothesis LDL is protected against oxidative stress by i.a. the Q10 antioxidants, thereby slowing down the formation of modified LDL. More specifically, the potent lipophilic antioxidants α-TOH (tocoferol, vitamin E), QH2-10 (ubiquinol-10, the reduced form of ubiquinone-10 or Q10), β-carotene and lycopene are supposed to be the important factors in this protection process. There is no unanimity about the relative physiological importance of these compounds.
The analysis by liquid chromatography with oxidative electrochemical detection (LC-EC) is very attractive because of its sensitivity and selectivity for the reduced compounds. However, due to the high lipophilicity of the compounds of interest very high modifier concentrations are required in the mobile phase putting specific demands to the LC-EC analysis to maintain sufficient electrical conductivity of the mobile phase. The method described here provides the conditions required. Detailed data regarding the method are given in [1].

Method

Blood samples were collected into evacuated tubes containing K3-EDTA and placed on ice in the dark. Within 2 hours the plasma was separated by centrifugation. Subsequently, ultracentrifugation was performed to isolate the LDL. Prior to extraction BHT (butylated hydroxytoluene) as antioxidant (250 µg/ml) was added and all treatments were done in a nitrogen atmosphere. Samples (200 µl) were mixed with 2 ml methanol, 4 ml hexane was added, vortex mixed, centrifuged, the hexane layer collected and the procedure was repeated.

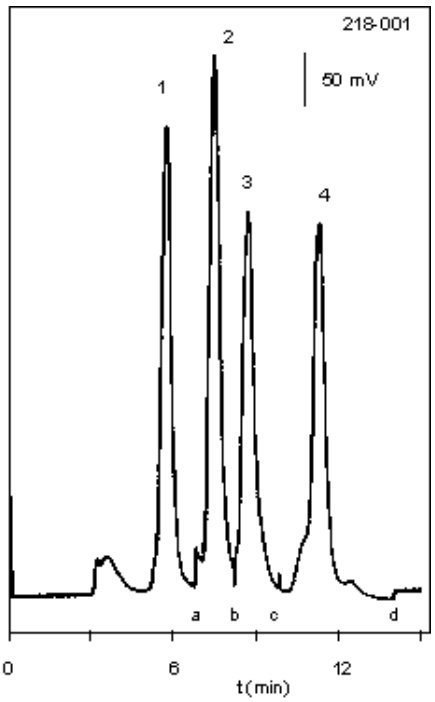


Fig. 1. Analysis of human LDL. Concentrations (amounts) are: 1. a-tocopherol (vitamin E) 3.6 µmol/l (72.9 pmol), 2. ubiquinol-9 0.77 µmol/l (15.5 pmol), 3. ubiquinol-10 0.21 µmol/l (4.2 pmol), and 4. b-carotene 0.11 µmol/l (2.2 pmol). The range is programmed at 10 (t=0), 1 (a), 0.5 (b), 1 (c) and 10 nA/V (d). Courtesy ref [1].

The 2 hexane layers were pooled, dried with nitrogen, the residue stored at –20°C and dissolved in mobile phase just prior to analysis. See [1] for further details.

References

1. Yolanda B. de Rijke et al., Arterioscler. Thromb. Vasc. Biol. 17 (1997) 127-133

Table 1

Set-up	
Column	Inertsil ODS-2, 5 µm, 200 x 3 mm
Flow rate	0.35 mL/min
Sample	20 µl extracted from human plasma
Mobile phase	22.5% methanol, 77.5 % ethanol/isopropanol (95/5), 20 mM LiClO4
Temperature	30 °C for column and flow cell
E-cell	600 mV vs. Ag/AgCl (sat'd with LiCl)

Recommendation

The advised configuration for this application is the ALEXYS Analyzer using an autosampler with sample cooling option.

PART NUMBERS AND CONFIGURATION	
180.0035C	ALEXYS Analyzer – cooled
110.4305	VT03 flow cell, 3 mm GC, HyREF

Fat soluble Vitamins A, D, E, K

Introduction

Vitamins A, D, E and K belong to the fat soluble vitamins. These vitamins are involved in several biochemical processes in the body. Briefly, **vitamin A** (retinol) plays a role in vision and bone growth. It comes from animal sources such as eggs, meat and dairy products. Beta-carotene, a precursor, comes from fruit and vegatables (paprikas, carrots).

Vitamin D (ergocalciferol, D2, cholecalciferol, D3) promotes the adsorption of calcium in the body, essential to development of bones and teeth. Vitamin D is made by the body when exposed to sun light, or via our diet of cheese, butter, fish, and milk.

Tocopherol, or **Vitamin E**, is an important antioxidant. Antioxidants protect cells against the effects of free radicals, which are potentially damaging by-products of the body's metabolism.

Vitamin K denotes a group of 2-methilo-naphthoquinone derivatives. They are needed for the posttranslational modification of certain proteins, mostly required for blood coagulation. Normally it is produced by bacteria in the intestines.

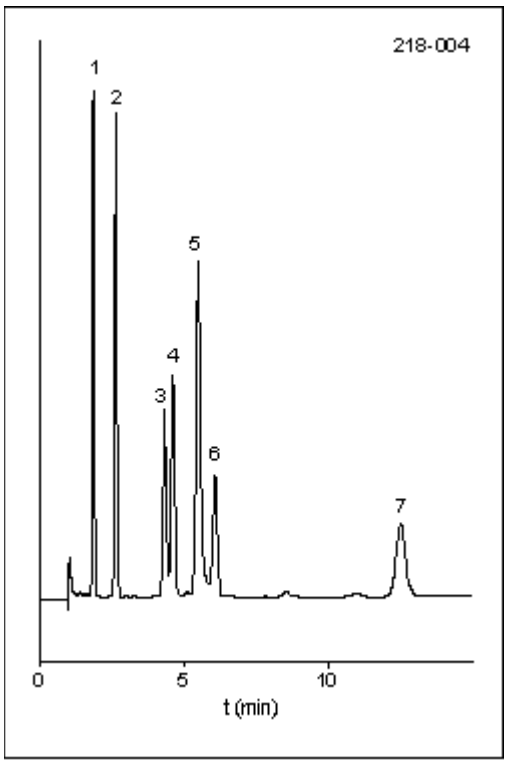


Fig. 1. Analysis of vitamin A (1), vitamin A acetate (2), vitamin D2 (3), vitamin D3 (4), vitamin E (5), vitamin K2 (6) and vitamin K1(7). Scale: 240 nA full scale.

Method

The HPLC system consists of reversed phase column and a apolar mobile phase (Table I). For EC detection an electrolyte is required therefore lithium perchlorate was added to the mobile phase. A VT03 cell with glassy carbon electrode has been used. Under these extremely apolar conditions the HyREF is the best choise for a reference electrode.

Only when Vitamin K must be analysed a second pre-reduction cell is applied.

Table 1

Set-up	
Column	Spherisorb ODS 100 x 4.6 mm, 3 µm
Flow rate	1.0 mL/min
Sample	Vitamins A and A acetate: 5 µM, E: 10 µM, D2, D3, K1, K2: 100 µM
Mobile phase	100 mmol/l lithium perchlorate, 96 % MeOH
Temperature	30 °C for column and flow cell
E-cell	1050 mV vs. Hy-REF, Only for vit K: pre-reduction: -400 mV

Recommendation

The advised configuration for this application is the ALEXYS Analyzer using an autosampler with sample cooling option.

PART NUMBERS AND CONFIGURATION	
180.0035C	ALEXYS Analyzer – cooled
110.4305	VT03 flow cell, 3 mm GC, HyREF